AN ASSESSMENT OF THE NEUROTOXIC POTENTIAL OF SPECIFIC PCB CONGENERS

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ABSTRACT

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PCBs have been an ideal industrial chemical due to their stable nature and resistance to heat but over time they have become significant environmental contaminants. The main emphasis in these studies has been to define the effects of PCBs on the morphology of the developing chicken brain using histological techniques and computed tomography. Experiments were also designed to assess the neurocytotoxicity of PCB congeners using the Fink-Heimer stain for degenerating axons and terminals. Experiments involved injecting domestic chicken (*Gallus domesticus*) eggs with individual PCB congeners. There were no significant differences in the amount of asymmetry present between treatment groups and the non-injected control group ($p \geq 0.05$). It was found that the normal development of asymmetry in avian neural systems as well as external forces involved in the development of the chick in the egg were likely significant enough to overshadow subtle changes brought on by exposure to environmental contaminants.

To Dale and my parents, without whom this work would not have been possible.

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LIST OF ABBREVIATIONS

AA	Archistriatum anterior
Bas	Nucleus basalis
BK	Beak
BO	Bulbus olfactorius
CA	Anterior commissure
CB	Cerebellum
CH	Cerebral hemispheres
CO	Chiasma opticum
E	Ectostriatum
Ey	Eye
FA	Tractus fronto-archistriaticus
FB	Frontal bone
FPL	Lateral forebrain bundle
HA	Hyperstriatum accessorium
HD	Hyperstriatum dorsale
Hp	Hippocampus
HV	Hyperstriatum ventrale
INP	Nucleus intrapeduncularis
LFS	Lamina frontalis
LFSM	Lateral frontalis suprema
LH	Lamina hyperstriatica
LMD	Lamina medullaris dorsalis
LPO	Lobus paraolfactorius
N	Neostriatum
NB	Nasal bone

nCPa	Nucleus commissurae pallii
NK	Neck
P	Pineal gland
PA	Paleostriatum augmentatum
PP	Paleostriatum primitivum
SL	Nucleus septalis lateralis
TSM	Tractus septomesencephalicus

INTRODUCTION

In this thesis I will present the results of three studies that attempted to assess the potential neurotoxic effects of polychlorinated biphenyls (PCBs). The introduction will discuss PCBs, the history of their use, and the importance of determining their effects on the central nervous system (CNS). The subsequent chapters will present different approaches used to define the potential effects of PCBs on the CNS.

Polychlorinated biphenyls are industrial products that were first synthesized in 1881 by reacting chlorine gas with biphenyls. They became commercially available in 1930 and were produced in the U.S. primarily by Monsanto Chemical Co. of St. Louis, Missouri. Polychlorinated biphenyls were sold in mixtures based on their chlorine content under the trade name Aroclor®. There are 209 individual PCB congeners, each given an International Union of Pure and Applied Chemistry (IUPAC) number from 1 to 209. Each congener is comprised of a biphenyl ring with a different number of chlorine atoms attached at the ortho, para, and/or meta positions (Erickson 1986).

Polychlorinated biphenyls are members of a larger class of compounds, the halogenated aromatic hydrocarbons. Both 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,4,7,8-pentachlorodibenzofuran (PCDF) are members of this class. Both of these compounds are known environmental contaminants and considered to be highly toxic (Safe 1984). Of the 209 PCB congeners, there are four that do not have chlorine atoms

at the ortho positions but have chlorine atoms at the para position and at least two meta positions enabling them to attain a coplanar form similar enough in structure to mimic the highly toxic behavior of both TCDD and PCDF (Safe 1984, Tanabe 1988, Rice and O'Keefe 1995) (Figure 1). It is important to clarify coplanar versus planar congeners as these terms are used interchangeably in the literature. The coplanar configuration of a PCB congener means that each phenyl ring can lie in the same plane because there are no chlorine atoms at the ortho position preventing such rotation (Rice and O'Keefe 1995). The term planar, although used by some in the literature in place of coplanar, does not describe the position of the two phenyl rings relative to one another and is therefore not accurate. The coplanar aromatic hydrocarbons are believed to exert their primary biologic effects through the AH-receptor (Swanson and Bradfield 1993, Nebert 1989). The AH-receptor has been implicated in the regulation of xenobiotic metabolism, chemical carcinogenesis, and the mechanism of toxicity of halogenated aromatic hydrocarbons (Swanson and Bradfield 1993). One of the ligands for the AH-receptor is TCDD (Nebert 1989). Any compound that can mimic the structure of TCDD, such as planar PCB congeners, can also act as a ligand for the AH-receptor.

Polychlorinated biphenyls are highly stable and heat resistant, making them ideal for use in industry. They have been used in paints, printing inks, coolants for motors, transformer fluids, hydraulic fluids, plasticizers and plastics, in caulking compounds, adhesives, and carbonless copy paper, and as extenders for pesticides (Peterle 1991). Due to their widespread use and stability they have become ubiquitous in our environment. In the early 1970s it became evident that PCBs were becoming environmental contaminants and that chronic exposure could result in hazards to human health. This lead Monsanto Chemical Co., the primary U.S. producer to voluntarily ban the sale of

PCBs. In 1976 with the development of the new U.S. Toxic Substances Control Act the manufacture of PCBs was banned. The ban, which became effective after implementation by the U.S. Environmental Protection Agency in 1979, prohibited the manufacture, processing, commercial distribution and use of PCBs except in totally closed systems (Rice and O'Keefe 1995). Despite this ban, PCBs still enter the environment from approved former use, incineration, illegal dumping in landfills, and atmospheric deposition. PCBs can also be produced unintentionally as by-products during the processing of other chemicals (Erickson 1986).

Polychlorinated biphenyls are highly lipophilic and show low biodegradability, which enables them to persist in fish, bird, non-human mammal, and human populations (Peakall and Fox 1987, Tanabe 1988). The persistence of PCBs is a concern due to their bioaccumulative and biomagnification potentials. PCBs tend to accumulate in adipose tissue (Safe 1984). As PCBs are consumed by an individual organism, their preferential storage in adipose tissue creates an accumulation of the compound that may not be excreted at the same rate at which the organism has been exposed. In a study by Bush *et al.* (1974), PCBs were added to the drinking water of adult hens to reproduce low-dose chronic exposure. Bush found that PCB levels built up faster than they declined and that at a mean of 18.7 weeks, the toxicity was five times greater than at a mean of 1.6 weeks of exposure.

The biomagnification of PCBs in the food chain is also of concern (Colburn *et al.* 1990). Organisms found at higher levels of the food chain will have higher concentrations of toxicants than organisms present at lower levels. This creates a situation where a small concentration of a toxicant can enter an ecosystem low in the food chain and be

found in several-fold higher concentrations in organisms such as fish-eating birds, mammals, or humans residing at higher levels of the food chain (Safe *et al.* 1987). Toxicant levels in the Great Lakes are high enough that there are provisional warnings for humans not to eat various fish species that have a high lipid content (Baumann and Whittle 1988). There is evidence to suggest that exposure to PCBs in fish-eating birds from the Great Lakes may result in decreased reproductive success due to embryotoxicity and teratogenicity (Kubiak *et al.* 1989, Peakall and Fox 1987, Baumann and Whittle 1988, Elliot *et al.* 1989).

When environmental samples such as water, soil, or fish are analyzed for their PCB content, the standard method employed is gas chromatography (Burse *et al.* 1983, Sawyer *et al.* 1995). However, these methods for measuring levels of PCBs do not take into account individual congeners and their individual potencies. Safe (1994) emphasizes the importance of studying individual PCB congeners and suggests that risk assessments not rely solely on the toxicity of known commercial mixtures of PCBs. When the concentration of individual PCB congeners found in environmental samples is compared to the concentration of those same congeners in commercial PCB mixtures, the concentrations are more varied than would be expected. It is for this reason that isolating individual congeners and assessing their potential toxicity is more important than studying PCBs in commercial mixtures.

PCBs have been shown to affect the liver, integument, and immune, reproductive, and nervous systems (Hansen 1987, Hoffman 1994, Safe 1994). Several investigators have focused on the nervous system with an emphasis on defining the behavioral and chemical effects of PCBs (Agrawal *et al.* 1981, Ericksson 1988, Seegal and Shain 1992).

However, relatively few studies have investigated structural changes that may occur due to PCB exposure. This thesis is comprised of three separate but related studies, each of which attempts to define the toxicological effects of individual PCB congeners by assessing structural changes in the central nervous system that may occur as a result of exposure.

In the first study, morphometric techniques were used to assess the effects of PCBs on the morphology of the cerebral hemispheres of hatchling domestic chickens (*Gallus domesticus*). Previous investigators (Henshel *et al.* 1995) have reported that exposure to a mixture of environmental contaminants including PCBs results in morphologic asymmetry of the cerebral hemispheres in hatchling great blue herons (*Ardea herodias*) and have suggested that this asymmetry may be a useful biomarker for contamination. The present study was designed to determine whether two environmentally relevant non-ortho substituted PCB congeners, 3,3',4,4'-tetrachlorobiphenyl (IUPAC #77) and 3,3',4,4',5-pentachlorobiphenyl (IUPAC #126), could cause morphologic asymmetry in the cerebral hemispheres.

The second study was a preliminary investigation into the potential usefulness of computed tomography (CT) to address the same issue of cerebral hemispheric asymmetry. Eggs of double-crested cormorants (*Phalacrocorax auritus*) from the Great Lakes were removed from the wild and injected with congener 126. This study was designed to determine if CT imaging could replace the need to sacrifice the bird in order to obtain information about the morphology of the cerebral hemispheres. If this were possible, the brains of wild birds could be measured *in situ* and the birds then returned to their habitats.

The third study employed the Fink-Heimer silver impregnation stain for degenerating axons and terminals to determine whether exposure to an ortho-substituted PCB congener, 2,2',5,5'-tetrachlorobiphenyl (IUPAC #52), or a coplanar PCB congener, such as congener 126, would result in axonal neuropathy. Work by Seegal and Shain (1992) has suggested that the ortho-substituted congeners, which are non-planar and therefore do not mimic the structure of TCDD, are responsible for the neurotoxic effects that have been observed in their research. They found that in non-human primates only the ortho-substituted congeners were detectable in the brain after sub-chronic exposure to a commercial mixture of PCB congeners. The same ortho-substituted congeners were responsible for reducing dopamine levels in both non-human primate brains as well as in pheochromocytoma cell cultures (PC12). In the present study, the Fink-Heimer silver stain was used to determine whether reductions in dopamine levels may be accompanied by signs of axonal and terminal degeneration.

Chapter 1

ASSESSMENT OF CEREBRAL HEMISPHERIC SYMMETRY IN HATCHLING CHICKENS EXPOSED *IN OVO* TO POLYCHLORINATED BIPHENYL CONGENERS

PCBs have been shown to affect the liver, integument, and immune, reproductive, and nervous systems of both avian and mammalian species (Hansen 1987, Hoffman 1994, Safe 1994). Many of the studies directed toward the central nervous system (CNS) have defined several behavioral and chemical effects of PCB exposure (Agrawal *et al.* 1981, Ericksson 1988, Seegal and Shain 1992). Ericksson (1988) suggested that PCB congener 77 (3,3',4,4'-tetrachlorobiphenyl) had an effect on the muscarinic cholinergic receptors in the mouse brain during development. Agrawal *et al.* (1981) reported that mice exposed *in utero* to PCB congener 77 showed a decrease in the number of dopamine receptor binding sites as well as low dopamine concentrations in the corpus striatum. A series of studies by Seegal and colleagues indicated that certain PCB congeners reduced dopamine concentration in the hypothalamus and basal ganglia of nonhuman primates as well as in pheochromocytoma (PC12) cell cultures (Seegal *et al.* 1991, Shain *et al.* 1991, Seegal and Shain 1992).

Recently, Henshel *et al.* (1995) reported that exposure to a mixture of environmental toxicants including PCBs may have an impact on the morphological

development of the CNS. They examined the brains of great blue heron hatchlings (*Ardea herodias*) from colonies in the Strait of Georgia, British Columbia, which were contaminated with a mixture of toxicants including polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and PCBs. They reported a grossly detectable asymmetry of the cerebral hemispheres and suggested a causal relationship between cerebral asymmetry and exposure to PCDDs and related compounds. They proposed that this asymmetry could be a useful biomarker for assessing the effects of PCDDs and related compounds on central nervous system development.

One of the contaminants that may have contributed to the cerebral asymmetry found in great blue heron chicks from these contaminated sites was PCBs. We wished to determine if PCBs alone might also induce a morphological asymmetry of the cerebral hemispheres. Accordingly, we initiated a study on the effects of individual PCB congeners on the morphology of the developing chicken brain. We chose to examine the effects of PCB congeners 77 (3,3',4,4'-tetrachlorobiphenyl) and 126 (3,3',4,4',5-pentachlorobiphenyl) based on the results of a study by Yamashita *et al.* (1993) who reported that these two congeners accounted for the majority of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-toxic equivalents in eggs of double-crested cormorants (*Phalacrocorax auritus*) and caspian terns (*Hydroprogne caspia*) collected from the Great Lakes basin. Similarly, Sanderson *et al.* (1994) reported that these PCB congeners were the largest contributors to the TCDD-toxic equivalents present in double-crested cormorant eggs from five colonies nesting on the Great Lakes.

The domestic chicken (*Gallus domesticus*) was chosen as an experimental subject because much of the research dealing with *in ovo* exposure to environmental contaminants has been done with this species (Brunstrom and Orberg 1982, Brunstrom and

Darnerud 1983, Brunstrom and Andersson 1988, Brunstrom and Lund 1988) and it is known to be very sensitive to PCBs, having a higher death rate upon exposure than several other species of ducks, geese, and gulls. In addition, the domestic chicken is very sensitive to the PCB coplanar congeners 77 and 126 (Brunstrom and Andersson 1988, Brunstrom and Lund 1988).

Materials and Methods

White Leghorn chicken eggs were obtained from the Michigan State University Poultry Science Teaching and Research Center. PCB congeners 77 and 126 (99.9% pure) were obtained from AccuStandard (New Haven, CT), and dissolved in an emulsion of egg yolk, lecithin (Merck; Darmstadt, Germany), peanut oil, and water (Brunstrom and Orberg, 1982). Eggs were divided into three treatment groups and two control groups. Eggs in two of the treatment groups received single injections into the yolk of either congener 77 (3.0 or 9.0 μg/kg egg) or congener 126 (0.3 or 0.9 μg/kg egg) in a volume of 1 ml/kg egg on day 0 of incubation. Eggs from the third treatment group were injected on day 0 with a combination of congener 77 (0.9 μg/kg egg) and congener 126 (0.8 μg/kg egg) in a volume of 1 ml/kg egg. The dosages used for the single injection groups were based on the LD_{50} values of the congeners (congener 77 = 8.8 μg/kg egg and congener 126 = 0.6 μg/kg egg) as determined in another study (Powell *et al.* 1996$_a$). The dosages in the combination treatment group corresponded to the concentrations detected in double-crested cormorant eggs collected on Tahaquamenon Island, the least contaminated site in a study by Yamashita *et al.* (1993). An additional group of eggs served as the non-injected control, whereas the last group received an

injection of vehicle only (1 ml/kg egg) made up of lecithin, peanut oil, and water (Brunstrom and Orberg, 1982).

The eggs were incubated in a Petersime incubator (Gettysburg, OH) at 37.5–37.7° C and approximately 65% relative humidity. The eggs were candled on days 4 and 11 of incubation to check for viability. Chicks hatched or were in the process of pipping by day 21, at which time they were removed from the incubator. They were weighed and their heads examined for signs of gross abnormalities. Chicks that had not completely pipped were removed from their eggs, weighed, and examined.

Following examination, all chicks were overdosed with an intraperitoneal injection of sodium phenobarbitol (150 mg/kg body wt; 1 ml/kg body wt) and perfused transcardially with a heparinized 10% formalin-saline solution using a 60cc syringe and 25-gauge hypodermic needle. All procedures related to brain removal, sectioning, and mounting were standardized in order to minimize variation caused by differences in processing methodologies. Immediately following perfusion of each chick, the calvaria were incised sagittally along the midline of the skull. This was followed by a cut made perpendicular to the midline at the level of the tentorium cerebelli. The skull was then peeled back to allow for maximum exposure to fixative. The brains were removed from the calvaria 24 hours after perfusion, weighed, and transected at the level of the rostral midbrain. The rostral part of the brain containing the hemispheres was weighed and placed in 10% formalin-saline followed by a 30% sucrose-formalin solution to allow for better sectioning of the neural tissue. Brains were then embedded in gelatin and placed in 30% sucrose-formalin prior to sectioning. The brains were serially sectioned on a freezing microtome in the coronal plane at a thickness of 40 μm. One out of every five

sections (approximately 200 μm apart) was stained with cresyl violet, a basophilic stain for Nissl substance (Figure 2).

The anterior commissure (CA) was chosen as an initial reference point to begin measurements of symmetry. For the brain section to be useful for subsequent measurements, the CA had to be visible crossing the midline in a single section and be evenly distributed within both hemispheres. This criterion was used to ensure that the brains had been sectioned perpendicular to the midline. Once the caudal-most section containing the CA had been identified, a series of 8 sections approximately 400 μm apart and located rostral to the CA were chosen for hemispheric measurements (Figures 3 & 4). The brain image was enlarged 20x and the hemispheres were outlined on graph paper with the aid of a microprojector (Figure 5). A line was drawn vertically between the enlarged hemispheres to highlight the true midline of the brain. A line perpendicular to the midline was drawn to mark the widest point of the hemisphere on both the left and right sides of the brain. The height of each hemisphere at its greatest extent was marked by drawing a line parallel to the vertical midline. Once these width and height points had been established, the actual distances were measured using a digital tablet and the Bioquant Image Analysis System.

To determine whether there was a difference in the amount of asymmetry present in the treatment brains when compared to the control brains, the absolute difference between the widths and heights of the right and left sides of the brain was used to perform a univariate split plot analysis of variance (Gill 1986). The least square mean values were computed from a general linear model procedure in the statistical software SAS (SAS Institute Inc., 1990). These data were then used to compare the amount of

asymmetry between treatment and control groups ($p \leq 0.05$). A first degree orthogonal polynomial contrast was performed to assess differences in width and height measurements at each individual section level. The level of statistical significance for this analysis was also $p \leq 0.05$.

Results

The mean values for the left-right differences for both the width and height measurements of each group are presented in Table 1.

When width measurements of the cerebral hemispheres in PCB treatment groups were compared to those of the non-injected control group, there were no statistically significant differences in the amount of asymmetry present ($p \geq 0.05$). However, width measurements for the vehicle-injected control group were significantly different from those of the non-injected control group ($p \leq 0.05$). There were no significant width differences in the amount of asymmetry present between any of the PCB treatment groups and the vehicle-injected control group ($p \geq 0.05$).

When height measurements of the cerebral hemispheres in PCB treatment groups were compared to those of the non-injected control group, there were no statistically significant differences in the amount of asymmetry present ($p \geq 0.05$). However, when height measurements of the cerebral hemispheres in PCB treatment groups were compared to those of the vehicle-injected control group, there were significant differences in brains from chicks exposed *in ovo* to 0.9 μg congener 126/kg egg and in brains from chicks exposed *in ovo* to 0.9 μg congener 77 plus 0.8 μg congener 126/kg egg ($p \leq 0.05$). No significant differences were found between the other treatment groups and the

vehicle-injected control group ($p \geq 0.05$), although height measurements for the non-injected control group were significantly different from those of the vehicle-injected control group ($p \leq 0.05$).

A significant linear trend was apparent in the amount of asymmetry present in each brain. The amount of asymmetry increased linearly as measurements were taken from the most caudal end of the brain at section 1 to the most rostral end of the brain at section 8 ($p \leq 0.05$). In testing the validity of this trend, data from all groups with the exception of the vehicle-injected group were pooled to increase the sample size [width $n = 56$, height $n = 50$] (Figure 6). Data were pooled since there were no statistically significant differences in the amount of asymmetry present in the PCB treatment groups and the non-injected control group. The vehicle-injected control group was not included in this analysis because the amount of asymmetry present in this group was significantly different from the amount present in non-injected controls.

Discussion

The results of this study indicate that a degree of asymmetry may normally be present in the cerebral hemispheres of newly hatched chicks and that *in ovo* exposure to individual environmentally relevant PCB congeners from day 0 of incubation does not appear to significantly exacerbate this asymmetry. In this respect, our results differ from those reported by Henshel *et al.* (1995) in which they found a dose-related difference in brain measurements from birds originating in colonies exposed to varying degrees of contamination. The difference between our results and those of Henshel *et al.* (1995) may be due to the type and concentration of neurotoxicants present. Henshel examined

eggs contaminated with a mixture of several environmentally relevant toxicants including PCDDs and PCDFs; PCBs comprised only a small part of the entire complement of toxicants present. It is possible that exposure to other toxicants such as the PCDDs may have resulted in the reported enhanced asymmetrical development or that the various toxicants acted synergistically to affect cerebral development. The two PCB congeners used in our study are, nevertheless, significant components in mixtures of Great Lakes environmental toxicants and were chosen based on data that indicated that congeners 77 and 126 were two of the three predominant coplanar congeners present in double-crested cormorant eggs obtained from contaminated sites along the Great Lakes (Yamashita *et al.* 1993).

The presence of asymmetry in brains of control chicks is not surprising since several structures in the avian brain, including the hypoglossal nucleus, the nucleus mesencephalicus lateralis pars dorsalis, and the thalamofugal visual pathway, have been shown to be anatomically asymmetrical (Bradshaw and Rogers 1993). Furthermore, this asymmetry has been shown to be an essential feature of avian embryogenesis, persisting into adulthood (Bradshaw and Rogers 1993). One of the systems that has been shown to develop asymmetrically, the thalamofugal visual projection, is one of two major visual pathways in the avian brain. Several investigators have found that in the domestic chicken, there are a greater number of thalamofugal projections in the right forebrain than in the left forebrain (Boxer and Sanford 1985, Rogers and Sink 1988, Rogers and Bolden 1991) and that this asymmetry extends from hatching through the first 21 post-hatch days (Rogers and Sink 1988). Whether this finding would translate into a gross morphological asymmetry is presently undetermined. Although a greater number of

thalamofugal projections in the right forebrain than the left forebrain suggests an underlying cellular substrate for the asymmetry noted in the Henshel *et al.* (1995) study and the present study, we did not test for sidedness with respect to the observed asymmetry, whereas Henshel reported a greater area of convexity associated with the left hemisphere than with the right.

In addition to intrinsic structural asymmetry, there are also extrinsic factors that may play a role in the formation of brain asymmetry. A chick embryo in the egg is positioned so that the left side of its head is tucked against its body, occluding the left eye, while the right side of its head is turned toward the shell (Bradshaw and Rogers 1993). As the chick continues to develop in its egg, it rapidly outgrows the available space and begins to press against the confining shell. The unossified skull may be susceptible to compression during this period, creating compression of the cerebral hemispheres as well. In addition, at hatching, the skulls of birds are not yet fully ossified, with complete ossification taking up to at least three months in some species (Pyle *et al.* 1987). This compression, along with the substantial amount of pressure exerted against the hard shell during pipping, may account for an initial asymmetric appearance of the cerebral hemispheres. Our finding of a trend of increasing asymmetry from the caudal end of the brain to the rostral end is consistent with the rostral parts of the skull being pressed against both the chick's body and the shell while in the egg. Both the intrinsic structural asymmetry and extrinsic molding forces may account, at least in part, for any significant differences in asymmetry found in our study.

It is also important to note that the domestic chicks used in this study are precocial and thus are further along in their development at hatch than are the altricial

species examined by Henshel *et al.* (1995). Accordingly, it might be expected that extrinsically induced forces brought on by a hard shell alongside a thinner and less ossified skull might result in a greater degree of cerebral hemispheric asymmetry in altricial species than in precocial species. In either case, it is apparent that an examination of the cerebral hemispheres for symmetry should extend beyond the newly hatched stage to the point when the hemispheres have had a chance to regain their normal configurations within partially or fully ossified skulls. Furthermore, an additional examination of symmetry beyond the newly-hatched stage may be useful in assessing the effects of any transient developmental asymmetries within the CNS, such as those induced by thalamofugal visual projections, on hemispheric measurements.

Finally, it should be noted that although every effort was made to standardize the methodology employed across chicks and extreme care was taken to ensure the orientation and accuracy of the measurements, it is possible that the structure used as the starting point for measurements was itself situated asymmetrically within the brain. We made the assumption that the anterior commissure develops in a symmetrical manner, crossing the midline in a plane precisely perpendicular to the midline in every brain. If this initial plane is itself asymmetrical, then subsequent perpendicular lines used for measuring will also be asymmetrical. This uncertainty would also apply to the use of surrounding bony or soft tissue structures for taking measurements.

In summary, the inherent asymmetry of the hatchling avian brain should be considered as a possible confounding factor when attempting to use cerebral asymmetry as a biomarker for environmental contamination. The normal development of asymmetrical CNS systems, as well as the presence of external molding forces, may

overshadow any subtle changes brought on by exposure to environmental neurotoxicants. The transient nature of both of these phenomena, as well as the accuracy of the measuring technique itself, should be taken into consideration when assessing the usefulness of asymmetry as a biomarker.

Chapter 2

COMPUTED TOMOGRAPHY (CT) AS A TOOL IN DETERMINING THE PRESENCE OF CEREBRAL HEMISPHERIC ASYMMETRY IN LIVE BIRDS

The present study was initiated to determine whether or not computed tomography (CT) would be a useful tool for investigating the question of cerebral asymmetry in birds. CT is an advanced imaging technique that uses x-rays and computers and can produce cross-sectional images of individual birds. In human medicine, CT has been used for diagnosing brain lesions caused by intracranial masses or fluid filled cavities, vascular lesions, and changes in ventricle size, ventricle:brain ratio, cortical atrophy, brain volume, and brain asymmetries associated with schizophrenia (Rubin *et al.* 1993).

In the previous study (Chapter 1), histological sections were used to measure asymmetry. One problem related to examining neural tissue is that birds must be killed in order to obtain measurements. This requirement may limit the scope of avian species examined since it is not always easy to obtain specimens from the wild and species of particular interest may be listed as threatened or endangered. The use of domestic chickens as surrogates for wild species is problematic for two main reasons. First, chickens may have different sensitivities to environmental toxicants than wild species. Second, as indicated in the previous chapter, chickens are precocial species, while a large

number of wild avian species are altricial relative to brain development. The differences in these two rates of CNS development may make comparisons between species difficult. There is also no risk that the process of histologically sectioning the brain could cause any physical alterations because CT sectioning does not cause any disruption of the tissues. CT-generated sections may also be more instructive than histologic sections in analyzing brain symmetry because the computer is able to reformat images in other planes (dorsal, sagittal) which could be used to determine further the symmetry of the entire brain. Based on these reasons, a preliminary study designed to assess the potential of CT as a useful technique in establishing the presence of brain symmetry was performed.

Materials and Methods

The eggs used in this study were from double-crested cormorants obtained from Lake Winnipegosis in Manitoba, Canada, a site considered to have relatively low levels of environmental contamination. Five eggs were injected with 100 μg PCB congener 126/kg egg in a vehicle of triolein. In a previous study (Powell *et al.* 1996$_b$), it was determined that the LD_{50} for congener 126 is 2.3 μg/kg egg in the domestic chicken. The higher concentration of congener 126 used in the present study was chosen because the double-crested cormorant is considerably less sensitive than the chicken. The higher dose was also used to maximize possible toxic effects on hemispheric symmetry. Five eggs were used as non-injected controls. The eggs were incubated in a Petersime incubator (Gettysburg, OH) at 37.5–37.7°C and approximately 65% relative humidity. The eggs were candled on days 4 and 11 of incubation to check for viability.

At hatching, the chicks were sedated with intraperitoneal injections of sodium phenobarbitol (75 mg/kg body wt; 0.5 ml/kg body wt) to allow examination with the CT scanning procedure, which took approximately five minutes for each bird. A GE 9800 CT scanner was used to obtain images. The birds were placed on their dorsa with their beaks tilted back in an attempt to obtain images in the same plane as the histological sections that would subsequently be produced from the same birds (Figure 7).

The CT procedure involves placing the bird on a table that advances into a gantry containing the x-ray tube, x-ray collimators, and x-ray detectors. The x-ray tube rotates 360° around the patient on the table and is the source of the x-rays. The x-ray collimator, which is located between the tube and the patient, determines the volume of tissue that will be represented in each image produced. The x-ray detector absorbs the x-ray photons emitted from the patient and converts them to an electrical signal. The computer uses this signal to produce a number that creates an image on the screen (Figure 8).

Each image produced by CT incorporated a volume of tissue 1.5 mm in thickness, the smallest interval obtainable with this machine. The computer screen on which the CT image was displayed had an underlying matrix. The matrix was composed of 512 columns and 512 rows, with each column or row designated as a consecutive number from 1 to 512. The columns comprised the x-axis and the rows made up the y-axis. Using this matrix, the midline of the brain section was associated with a number on the x-axis (Figure 9). The same point on the x-axis was assumed to be at the midline of all sections of the same brain. Once the midline was identified, the cursor was moved to the widest point of the left hemisphere. The computer measured the distance between those two points in centimeters (Figure 10). The same procedure was repeated for the

right hemisphere. The absolute difference between the left and right hemispheres was recorded. For the height measurement, the cursor was placed along the y-axis at a point on the dorsal-most surface of the left hemisphere. The cursor was then placed along the ventral-most surface of the left hemisphere and the computer measured the distance between those two points in centimeters (Figure 11). The same procedure was repeated for the right side of the brain. The absolute difference in height measurements between the left and right hemispheres was recorded.

A range of Hounsfield units or CT numbers was chosen to differentiate bone and connective tissue from neural tissue. The cursor was placed over what was obviously neural tissue, bone, or connective tissue, and the respective CT numbers generated by the computer were recorded. Since there are no predetermined CT numbers for the immature neural tissues of an altricial avian species, a range of numbers had to be chosen. Any number that was generated while the cursor was overlying neural tissue but not generated when overlying non-neural tissue was used in the chosen range.

Once the hatchlings had been scanned they were euthanized with sodium phenobarbitol (150 mg/kg body wt; 1 ml/kg body wt), perfused, and their brains prepared for histological analysis as described in the previous study (Chapter 1).

Results and Discussion

The results of the CT measurements are presented in Table 2. These data were not statistically analyzed due to the discovery of several limitations that affected the reliability and usefulness of CT as a method for assessing cerebral symmetry. The first issue that arose was the range of CT numbers chosen to designate neural tissue. In order to understand the numerical designation, it is necessary to provide a brief background on

CT concepts and methodology (Hathcock and Stickle 1993). The CT image is composed of several tiny squares called pixels. Each individual pixel is a two-dimensional representation of a three-dimensional block of tissue. The color assigned to each pixel, black, white, or shades of gray, is based on the type of tissue that is being represented in that pixel. The color in the pixel is determined by how much radiation is attenuated by each volume of tissue. Each tissue type will attenuate the x-ray beam differently based on its atomic number and electron density. The x-ray attenuation of each tissue type is quantitatively measured and termed the linear attenuation coefficient. The computer calculates the linear attenuation coefficient of the tissues in each pixel and assigns a CT number to that pixel. CT numbers range from +1000 to -1000, with cortical bone being +1000, air being -1000, and water being zero. All other tissues are assigned numbers relative to the densities of bone, air, and water.

There is normal variation in the densities of both bone and soft tissue within the same bone or soft tissue organ (Owens 1982). Variations in the density of bone are due to differing ratios of compact to spongy bone, trabecular bone to intertrabecular spaces, and cortex to medullary canal. The differences in soft tissue densities arise from variations in volume, thickness, or the degree of compactness of the tissue in question. It is due to these reasons that CT numbers for any given species may not apply to the same tissue in a different species. The present study has extended this finding of variability by suggesting that CT numbers assigned to developing tissues within a single species may also vary significantly from those reported for adult tissues in the same species. This finding results in several problems associated with interpretation of numerical CT data. In an altricial avian species, such as the double-crested cormorant, the birds hatch at a very early developmental stage, whereas precocial species hatch at

a significantly later stage of development. In either situation, the brain at these early stages of development has not yet completed its neural growth. The overall density of the brain is constantly changing until maturity. Structures which are not mature at hatch and contribute to the overall density of the brain, include axons, dendrites, myelin sheaths, and glial cells. Thus the neural tissue being measured will differ in density based on the different rates of maturation for altricial and precocial species. Likewise, the structures surrounding the brain such as connective tissue and bone have not reached their full density and cannot be compared with adult CT numbers.

Another problem that became apparent during the course of this study is that surrounding immature non-neural tissues, such as the non-ossified skull, may attenuate the x-ray beam to the same degree as the immature neural tissue. In this case, it was difficult to differentiate with any certainty the CT numbers for brain from those for connective tissue or bone. When the cursor was placed over an area in the image that was clearly neural tissue, the CT number was the same as when the cursor was placed over an area that was clearly not neural tissue. Because of this, a range of CT numbers had to be defined. The range chosen was between 55 and 70 and was based on CT numbers most consistently found in an area that was obviously neural tissue. Difficulty arose when the cursor was placed over an area that could have either been neural tissue or connective tissue and the CT number fell both in and out of the chosen range on different instances. Although a standard index of CT numbers should be developed so that specific tissue types can be delineated from one another at specific developmental stages, it is unclear how best to deal with differentiating dissimilar tissue types when the CT numbers overlap.

Another critical point is that the measurements of the cerebral hemispheres can be artificially skewed if the birds are not positioned correctly on the scanning table. Improper positioning resulted in only four of the ten brains being useable for measurements. Part of the problem was that CT images were not viewed while the bird was still on the table. Viewing the images immediately as they were produced, while the birds were still on the scanning table, would have provided the information needed for correct positioning. Another way to ensure symmetric positioning would be to place the birds in a stereotactic apparatus on the scanning table. The use of extracranial structures such as the frontal bone, nasal bone, and eyes could be used as guides during CT scanning to determine if the birds have been positioned symmetrically. In this study, extracranial structures were used after the images were taken to determine whether or not positioning was symmetrical (Figures 12 & 13). Since the goal of this study was the investigation of asymmetry, proper symmetrical positioning was critical for success.

The birds were sacrificed after CT scanning and perfused so that their brains could be measured using the morphometric technique described in Chapter 1. It was found after the brains had been sectioned that they would not be suitable for morphometry. The fixation protocol used in the previous study was appropriate for a precocial avian species (domestic chicken) whose neural development is at a more advanced stage at hatching than an altricial species (double-crested cormorant). It was apparent that for an altricial species the fixation period should have been extended. The brains were too soft when they were sectioned and therefore led to deformed sections when they were placed on glass slides. This created a grossly artificial asymmetry.

These preliminary studies have revealed several important considerations and limitations concerning the use of CT in evaluating the symmetry of the developing brain.

First, in order for CT imaging to be useful in addressing the issue of symmetry, standard CT numbers have to be defined for the neural tissue under consideration so that there is confidence that only neural tissue is being measured. Measurements may have to be made at different times during development to take advantage of changes in density occurring during maturation and differentiation of neural and adjacent structures. Second, proper positioning is necessary. The advantage of CT over the histologic technique is that proper positioning can be corrected while the bird is on the scanning table if the images are viewed immediately. Third, assessment of histological sections in altricial species requires longer post-fixation times to allow for better preservation of neural tissue. Finally, CT cannot differentiate specific nuclei or tracts undergoing proliferation or degeneration. If determination of effects at the cellular level is required, it would still be necessary to sacrifice the birds to obtain histological sections. Until these points are addressed, CT may not be as effective a tool in defining developmental abnormalities of the CNS as initially hoped.

Chapter 3

ASSESSMENT OF THE NEUROCYTOTOXIC POTENTIAL OF BOTH A COPLANAR AND NON-PLANAR PCB CONGENER USING THE FINK-HEIMER STAINING TECHNIQUE

In the previous two studies (Chapters 1 and 2) the possible effects of coplanar PCB congeners on the gross morphometry of the cerebral hemispheres in two avian species were examined. The experiments in the present chapter were designed to determine the possible cytotoxic changes caused by exposure to either a coplanar or non-planar ortho-substituted PCB congener.

There is growing evidence to indicate the existence of two classes of PCBs differentiated by the presence or absence of neurologic effects. In 1986, Shain and colleagues exposed animals to complex mixtures of PCBs in their feed and analyzed the brain tissue, using a congener specific gas chromatography technique, to determine which congeners accumulate in the brain. They found that several ortho-substituted, non-planar congeners were found in the brain at concentrations as much as 100-fold greater than their concentrations in the adulterated feed, while there were no detectable concentrations of coplanar congeners in the brain. In a comprehensive study, Seegal and Shain (1992) demonstrated that ortho-substituted congeners such as congeners 28, 52, and 47 (2,4,4'-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl, and 2,2',4,4'-tetrachlorobiphenyl,

respectively), caused a reduction in dopamine content in both non-human primate brains and pheochromocytoma (PC12) cells. Reductions in brain concentrations of dopamine have been found in the caudate nucleus and substantia nigra and it has been suggested that the reduction in dopamine levels was caused by an inhibition of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis (Seegal *et al.* 1991). The coplanar congeners such as those used in the two previous studies of this thesis had no effect on dopamine content.

The specific aim of this study was to determine whether an ortho-substituted, non-planar PCB congener shown to result in a reduction in dopamine content would also cause detectable signs of axonal and terminal degeneration in pathways that utilize dopamine as a neurotransmitter. In addition, brains exposed to congener 126 were analyzed to determine if congener 126 had cytotoxic effects as well and if the brain regions affected were the same as for congener 52. Finally, measurements were made of cerebral hemispheres in birds exposed to congener 52 to determine whether exposure to a non-planar congener might result in cerebral asymmetry at the time of hatch.

Materials and Methods

White Leghorn chicken eggs were obtained from the Michigan State University Poultry Science Teaching and Research Center. PCB congener 52 (2,2',5,5'-tetrachlorobiphenyl) and congener 126 (3,3',4,4',5-pentachlorobiphenyl), both 97-99% pure, were obtained from ULTRA Scientific (North Kingstown, RI). The congeners were dissolved in an emulsion of egg yolk, lecithin (Merck; Darmstadt, Germany), peanut oil, and water (Brunstrom and Orberg, 1982). Eggs were divided into two treatment groups and two control groups. Eggs in one of the treatment groups received

single injections of congener 52 at three different dosages (12.6, 6.3, or 3.1 μg/kg egg). The dosages for congener 52 were based on a study by Seegal and Shain (1992) in which they conducted a long-term feeding trial using Aroclor 1016, at dosage levels of 0.8, 1.6, or 3.2 mg/kg per day in non-human adult primates. They found that of the 27 congeners present in Aroclor 1016, only three were detected in the brain (ortho-substituted congeners 52, 28, and 47). In the Aroclor 1016 mixture, congener 52 comprised 2% of the total PCBs present, whereas in the brains, congener 52 comprised 14% of the total congeners detected. Furthermore, the EC_{50}, or effective concentration at which 50% of the dopamine content was reduced in PC12 cell cultures, was found to be 86 μM for congener 52. Using these numbers, the dosage required for 14% of congener 52 to accumulate in the brain at a concentration of 86 μM (22.2 mg/L) was calculated. The calculated dose was 6.3 μg/kg egg. The low dose used for egg injection was half the calculated dose (3.2 μg/kg egg) and the high dose used for egg injection was twice the calculated dose (12.6 μg/kg egg). Another group of eggs received a single injection of congener 126 at the highest dose used previously in the asymmetry study in Chapter 1 (0.9 μg/kg egg). One control group received single injections of the vehicle alone, whereas a second control group consisted of non-injected eggs. Table 3 lists the sample sizes and dosages for congener 52. All injections were made into the yolk on day 0 of incubation. Eggs were incubated in a Petersime incubator (Gettysburg, OH) at 37.5-37.7°C and approximately 65% relative humidity, and candled on days 4 and 11 of incubation to check for viability.

The chicks hatched or were in the process of pipping by day 21, at which time they were removed from the incubator. They were weighed and their heads examined

for signs of gross abnormalities. Chicks that had not completely pipped were removed from their eggs, weighed, and examined. All procedures related to brain removal and sectioning were exactly as described in Chapter 1. Once the brains were sectioned (40 μm thick), one out of every five sections, 200 μm apart, was stained with the Fink-Heimer silver stain for degenerating axons and terminals (Tanaka 1976). Adjacent sections in brains exposed to congener 52 were stained with cresyl violet and the sections handled exactly as described in Chapter 1 for the determination of symmetry in the cerebral hemispheres. The statistical procedures used in Chapter 1 to assess asymmetry were repeated for the congener 52 brains.

Results and Discussion

Results of hemispheric measurements indicate that no significant differences in hemispheric symmetry were present between the congener 52 treatment groups and the vehicle-injected or non-injected control groups ($p \geq 0.05$) (Table 3). The same significant linear trend was apparent in the congener 52 injected groups with the amount of asymmetry increasing linearly as measurements were taken from the most caudal end of the brain at section 1 to the most rostral end of the brain at section 8 ($p \leq 0.05$).

All brains from chicks exposed to either congener 52 or 126 were negative for the presence of axonal or terminal degeneration. In the avian brain, areas such as the paleostriatum primitivum, paleostriatum augmentatum, and lobus paraolfactorius are believed to be homologous to the mammalian dopaminergic centers. Therefore, particular attention was directed toward these sites as previous investigators have reported a significant reduction of dopamine levels in homologous areas in mammals (Seegal *et*

al. 1991). There are several possible explanations for these negative results, the simplest of which is that the dosages chosen may have been too low. Another possibility is that the exposure time was not long enough. In a study by Seegal *et al.* (1994), it was found that after exposure of Wistar-derived rats to 30 ppm of Aroclor 1254 from gestational day 8 until post-natal day 300, the number of tyrosine hydroxylase-containing neural cells was reduced by 46%. In the present study, exposure to PCBs extended only through 21 days of development in the egg and not beyond hatching. This period may not be long enough or perhaps exposure during the post-hatch period may be necessary for the full effects of PCBs as a neurotoxicant to be detected. Similarly, work by Tanaka *et al.* (1994) with triphenyl phosphite (TPP) and the development of the visual system in European ferrets has suggested that immature neurons are initially not susceptible to the effects of the neurotoxicant and neuropathy can be found only after there has been some degree of maturation of visual system neurons and connections. In the present study, chicken brains were not assessed beyond hatching. Whether a neuropathy would have been detectable later during CNS maturation is unknown.

Several investigators, using congener specific gas chromatography, have found that many PCB congeners accumulate in the brain (Seegal and Shain 1992, Ness *et al.* 1994). However, an accumulation of a specific congener does not necessarily translate into a cytotoxic effect. In this study, the intent was to determine the presence of cytotoxicity as a result of exposure to individual PCB congeners. The dosages for congener 52, an ortho-substituted non-planar congener, were determined based on information obtained from studies in non-human primates that had been exposed through feed. In this study, the chicks were exposed during their development in the egg.

Obviously there are differences related to how the PCB compounds are absorbed and metabolized by a developing chick and a non-human primate. It was not determined in this study whether or not the PCB congener accumulated in the brain to the concentration that had been calculated based on the study by Seegal and Shain (1992). Seegal *et al.* (1991) also reported that, when several congeners were mixed together prior to administration, a synergistic effect was produced that reduced dopamine content in non-human primate brains and in PC12 cells to a greater degree than when each congener was administered individually. This finding suggests that it is important not only to assess the neurotoxic potential of individual PCB congeners, but also to examine the neurotoxic potential of complex PCB mixtures found in the environment. Since an individuals exposure to environmental PCBs is usually as a mixture of congeners, the potentially neurotoxic effects of synergism should be taken into account when discussing the toxicity of individual congeners. It may be misleading to state that individual congeners do not have substantial toxic effects when synergistic toxic effects have not been taken into account.

Chapter 4

CONCLUSIONS

The experiments described in this thesis have attempted to define the potential neurotoxic effects of individual PCB congeners on the developing brain using both histologic and computer-imaging techniques. A protocol was developed to address the morphometric changes in the cerebral hemispheres that had been hypothesized to occur as a result of exposure to environmental contamination (Henshel *et al.* 1995). Computed tomography (CT) and the Fink-Heimer staining technique designed to reveal the presence of neurotoxicant-induced axonal and terminal degeneration were two other methodologies employed to determine the neurotoxicity of specific PCB congeners.

The major rationale for these experiments was based on a study by Henshel *et al.* (1995) in which it was found that exposure to certain environmental contaminants correlated with the development of asymmetrical cerebral hemispheres in hatchling great blue heron chicks. They proposed that this hemispheric asymmetry could be useful as a biomarker for environmental contamination. Unfortunately they did not consider other explanations for their findings. The normal development of asymmetrical CNS systems, as well as the presence of external molding forces, such as an unossified skull at hatching and a hard-shelled egg in which to develop, may overshadow any subtle changes brought on by exposure to environmental neurotoxicants. The transient nature of these events

should have been taken into consideration when assessing the usefulness of asymmetry as a biomarker.

The protocol developed in this thesis to assess the symmetry of the cerebral hemispheres was more exacting than that used in the Henshel study. First, the brains used in the Henshel study had been stored for varying periods of time and were not all processed in the same manner. In our study, all procedures related to euthanasia, brain removal, fixation, tissue sectioning, mounting, and staining were standardized in order to minimize variation caused by differences in processing methodologies. The brains were measured grossly with an engineering ruler in the Henshel study, whereas we used a digitizing morphometry computer system to obtain our brain measurements. The absolute differences in the right and left hemisphere measurements for both of these studies ranged from 0.05 mm up to 1 mm. Since these numbers are so small relative to the overall dimensions of the brain, it is critical that measurements be made with as much accuracy as possible. Based on the results of this study, it appears that cerebral hemispheric asymmetry is a complex multi-factorial phenomenon, which may not be as strongly correlated with environmental contamination as had been previously suggested (Henshel *et al.* 1995). As Bradshaw and Rogers (1993) so eloquently stated: "Given the growing list of both functional and structural asymmetries in birds, one begins to wonder whether it is not asymmetry but symmetry which, when it occurs, needs to be explained."

Based on our results, more preliminary research into the applicability of CT technology to address the issue of symmetry in cerebral hemispheres should be done. The major problem that developed during this study was the inability to positively identify neural tissue from other surrounding tissues in the immature bird. It was not

apparent at the time of the study that differentiating tissues based on CT numbers would be so difficult and that the CT numbers given to different tissues would overlap to such a degree. One solution to this problem is to develop standard CT numbers for the tissue type of the particular species involved. However, this may not be realistic, as it might be cost prohibitive. More importantly, it may be that the use of CT is more appropriate as an initial screen for neurotoxicity and that detailed neuropathological changes might still require the use of standard neurohistological techniques. At this time, use of CT as a diagnostic tool is best reserved for gross pathology such as that caused by intracranial masses or fluid filled cavities.

Finally, the last set of experiments involved the use of one PCB congener from both the neurologic and non-neurologic classes of PCBs. Using the Fink-Heimer staining technique the brains were evaluated for the presence of axonal and terminal degeneration. Although the results were negative, a closer examination of dosages, exposure times, and ages at which exposure is present is necessary before any definitive statements can be made regarding PCBs and morphologically characterized cytotoxicity.

APPENDICES

APPENDIX A

Tables 1–3

APPENDIX A

Table 1. Mean values (mm) for the absolute differences in width and height measurements between left and right cerebral hemispheres of hatchling domestic chickens exposed at day 0 of incubation to individual PCB congeners

Treatment[a] (μg/kg egg)	Mean values (mm) for measurements ± SE	
	Width (n)[b]	Height (n)
NIC	0.19 ± 0.04 (19)	0.14 ± 0.02 (16)
VIC	0.32 ± 0.04 (13)*	0.23 ± 0.02 (11)*
3.0 #77 (LD_{50} = 8.8)	0.31 ± 0.08 (4)	0.13 ± 0.04 (3)
9.0 #77	0.15 ± 0.09 (3)	0.18 ± 0.04 (3)
0.3 #126 (LD_{50} = 0.6)	0.27 ± 0.05 (8)	0.17 ± 0.03 (7)
0.9 #126	0.25 ± 0.04 (12)	0.16 ± 0.02 (13)**
0.9 #77 & 0.8 #126	0.23 ± 0.06 (6)	0.13 ± 0.06 (5)**

[a] NIC = non-injected control eggs, VIC = vehicle-injected control eggs, #77 = PCB congener 77 (3,3',4,4'-tetrachlorobiphenyl) and #126 = PCB congener 126 (3,3',4,4',5'-pentachlorobiphenyl).

[b] Differences in sample sizes for each measurement are due to the presence of cutting artifact. In order for the brain to be used for measurements, all cortical layers must have been intact. Loss of cortical tissue occurred more frequently in the height plane than the width plane.

* The amount of asymmetry present was significantly different when compared to that of the non-injected control groups ($p \leq 0.05$).

** The amount of asymmetry present was significantly different when compared to that of the vehicle-injected control groups ($p \leq 0.05$).

Table 2. CT numbers obtained from brain images of double-crested cormorants exposed to 100 μg PCB congener 126/kg egg at day 0 of incubation

	Mean values (cm) for the absolute difference in measurements	
Bird[a]	width	height
1	.04	.02
2	.02	.05
3	.03	.02
4	.08	.03

[a] All birds scanned were from the PCB congener 126 injected group. None of the non-injected birds were positioned correctly, therefore measurements were not taken

Table 3. Mean values (mm) for the absolute differences in width and height measurements between left and right cerebral hemispheres of hatchling domestic chickens exposed at day 0 of incubation to PCB congener 52

	Mean values for measurements ± SE	
Treatment (μg/kg egg)	Width (n)[b]	Height (n)
NIC	0.19 ± 0.04 (19)	0.14 ± 0.02 (16)
VIC	0.32 ± 0.04 (13)*	0.23 ± 0.02 (11)*
3.1 #52	0.41 ± 0.05 (8)	0.20 ± 0.03 (7)**
6.3 #52	0.27 ± 0.05 (8)	0.21 ± 0.21 (7)*
12.6 #52	0.22 ± 0.05 (10)	0.17 ± 0.02 (9)

[a] NIC = non-injected control eggs, VIC = vehicle-injected control eggs, #52 = PCB congener 52 (2,2',5,5'-tetrachlorobiphenyl)

[b] Differences in sample sizes for each measurement are due to the presence of cutting artifact. In order for the brain to be used for measurements, all cortical layers must have been intact. Loss of cortical tissue occurred more frequently in the height plane than the width plane.

* The amount of asymmetry present was significantly different when compared to that of the non-injected control groups ($p \leq 0.05$).

** The amount of asymmetry present was significantly different when compared to that of the vehicle-injected control groups ($p \leq 0.05$).

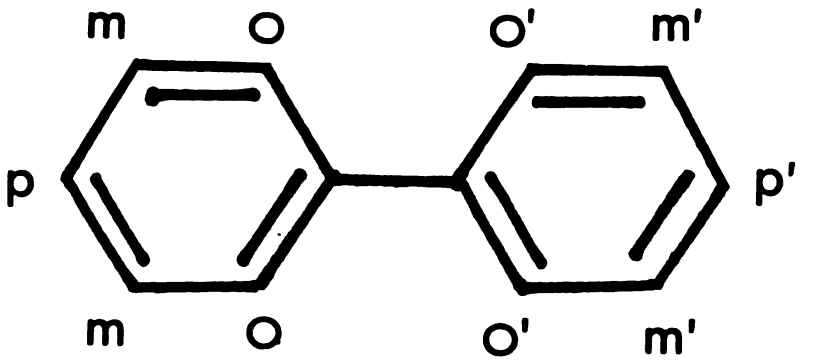
APPENDIX B

Figures 1–13

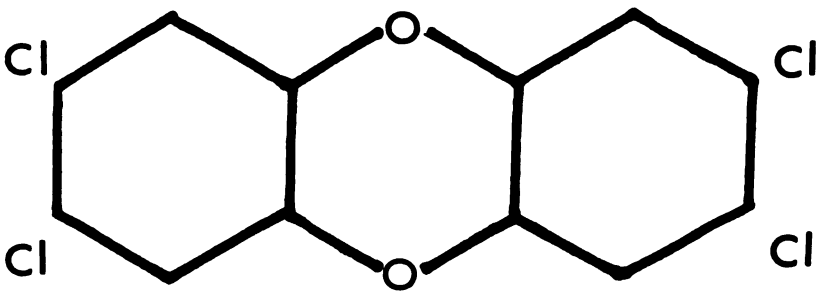
APPENDIX B

Figure 1. Structure of PCB congeners and comparison of structure to TCDD

Chlorine atoms can be distributed on the ortho (o, o'), meta (m, m'), or para (p, p') positions of the biphenyl structure. Those congeners that do not have chlorine atoms on the ortho (o,o') positions but do have chlorine atoms at the para positions and at least two meta positions attain a structure similar to TCDD. This enables them to mimic the highly toxic behavior of TCDD.



PCB



TCDD

Figure 2. Photomicrograph of a 40 μm thick section of a hatchling domestic chicken brain from the non-injected control group illustrating a cerebral hemisphere stained with cresyl violet

A. This section is taken through the cerebral hemispheres of a newly hatched chick at the level of the anterior commissure (level 1) magnification x 41. The boxed area indicates the area shown in plate B. B. A higher power photomicrograph of the boxed area in A illustrating the symmetrical orientation of the anterior commissure (between arrowheads). Magnification x 127. Note abbreviations on page viii.

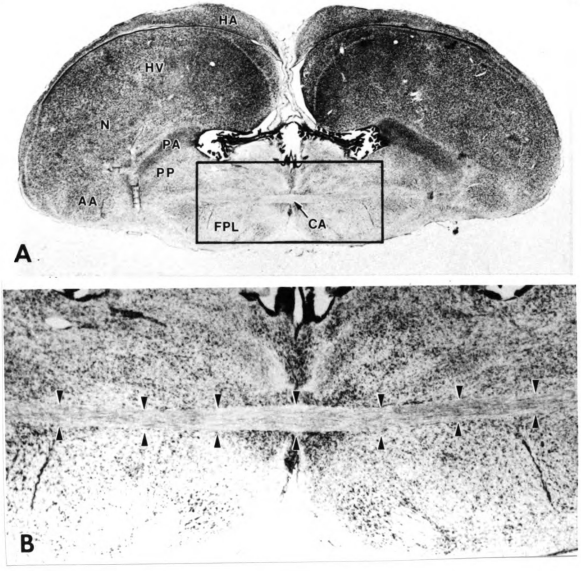


Figure 3. Line drawing of a parasagittal section through the chicken brain illustrating the levels from which each of the 8 sections were taken for hemispheric measurements

The numbers correspond to sections 1 to 8. Note abbreviations on page viii.

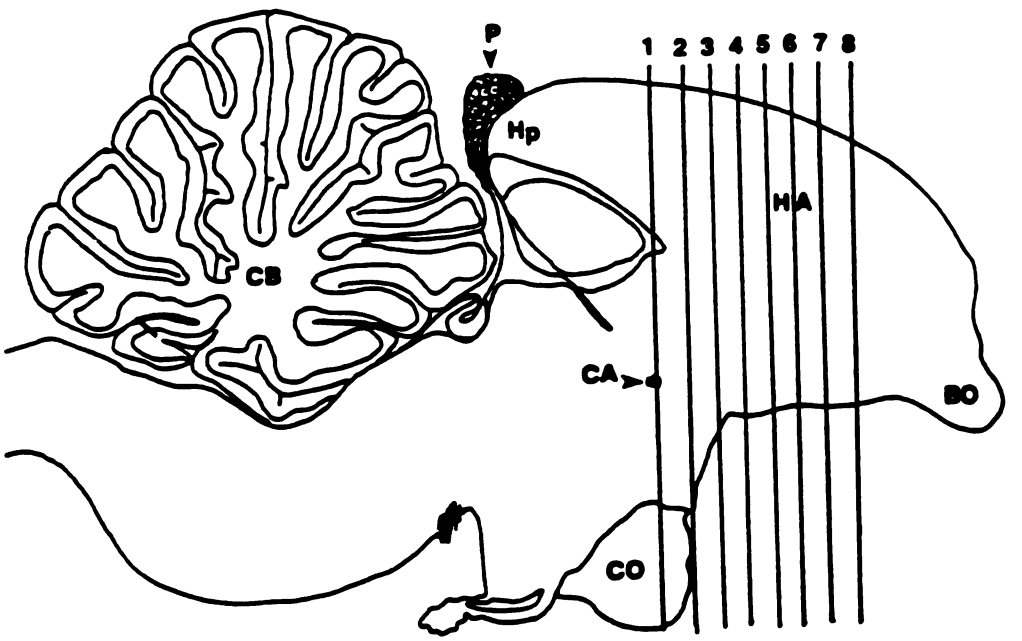


Figure 4. Line drawing of coronal sections at the eight section levels from which measurements for asymmetry were obtained from the brains of all PCB treatment and control groups of hatchling domestic chickens

Section 1 is located most caudally at the level of the anterior commissure and section 8 is located most rostrally. Nuclei and tracts were identified with the aid of an atlas (Kuenzel 1988). Note abbreviations on page viii.

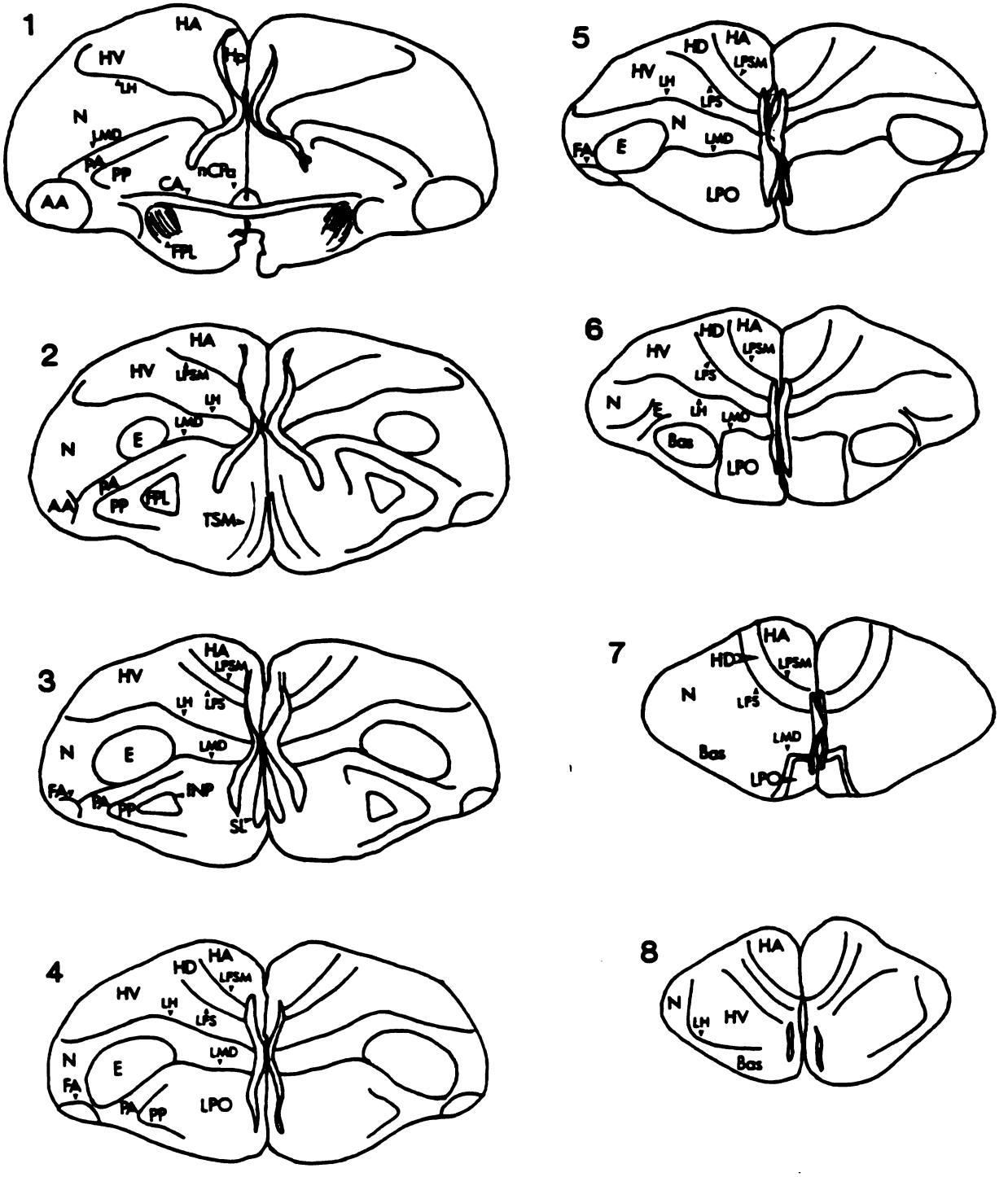


Figure 5. Line drawing of section level 1 showing how width and height measurements were obtained from a hatchling domestic chicken brain for all PCB treatment and control groups

Using an enlarged image of the cerebral hemispheres, this drawing illustrates a section at the level of the anterior commissure. The line drawn between the hemispheres indicates the true midline. Line AB, drawn perpendicular to the midline, denotes the widest point of the hemisphere. Line CD, drawn parallel to the midline, illustrates the maximum height of the hemisphere.

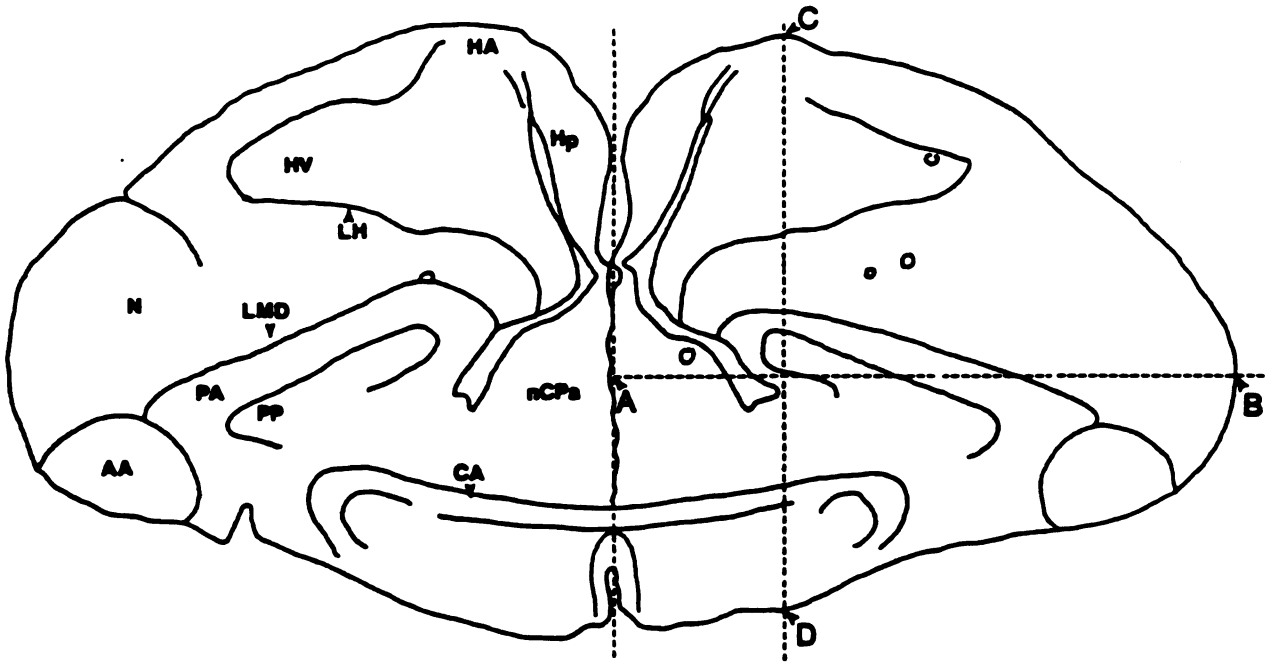


Figure 6. Histogram of width and height measurements by section level for all PCB treatment groups and the non-injected control group of hatchling domestic chickens

The absolute differences in width and height measurements at each section level for all groups except the vehicle-injected control groups. At each section level n=56 for the width measurements (total measurements = 448), and n=50 for the height measurements (total measurements = 400). Note the trend toward an increase in the amount of asymmetry as the sections move from the most caudal end to the most rostral end of the brain.

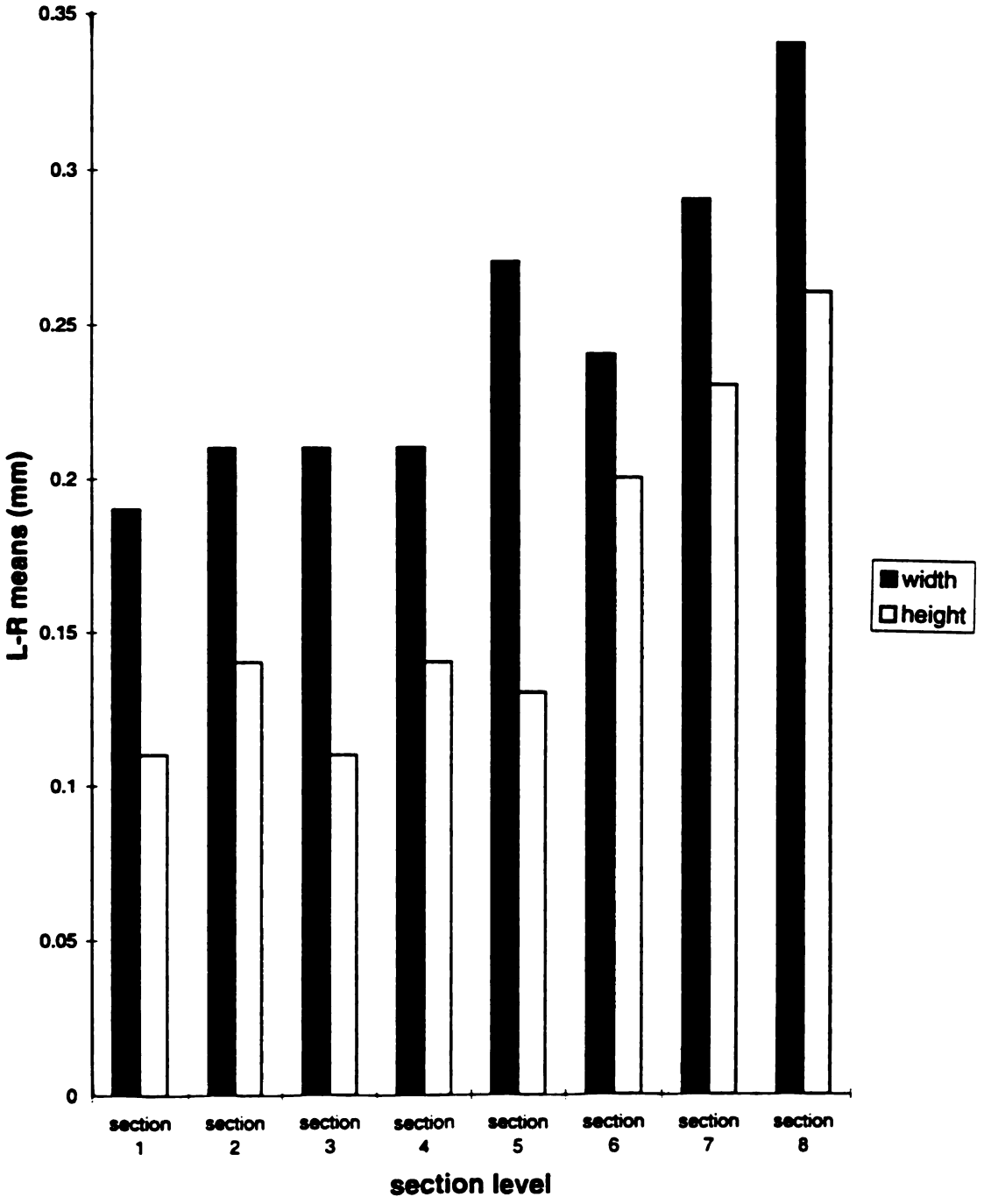


Figure 7. Comparison of bird brain positioning on computed tomography (CT) scanning table and histological sectioning

The bird's beak was pulled rostrally and the brain oriented in a position so that the CT images produced would be in the same plane as the histologic sections would be cut on the microtome.

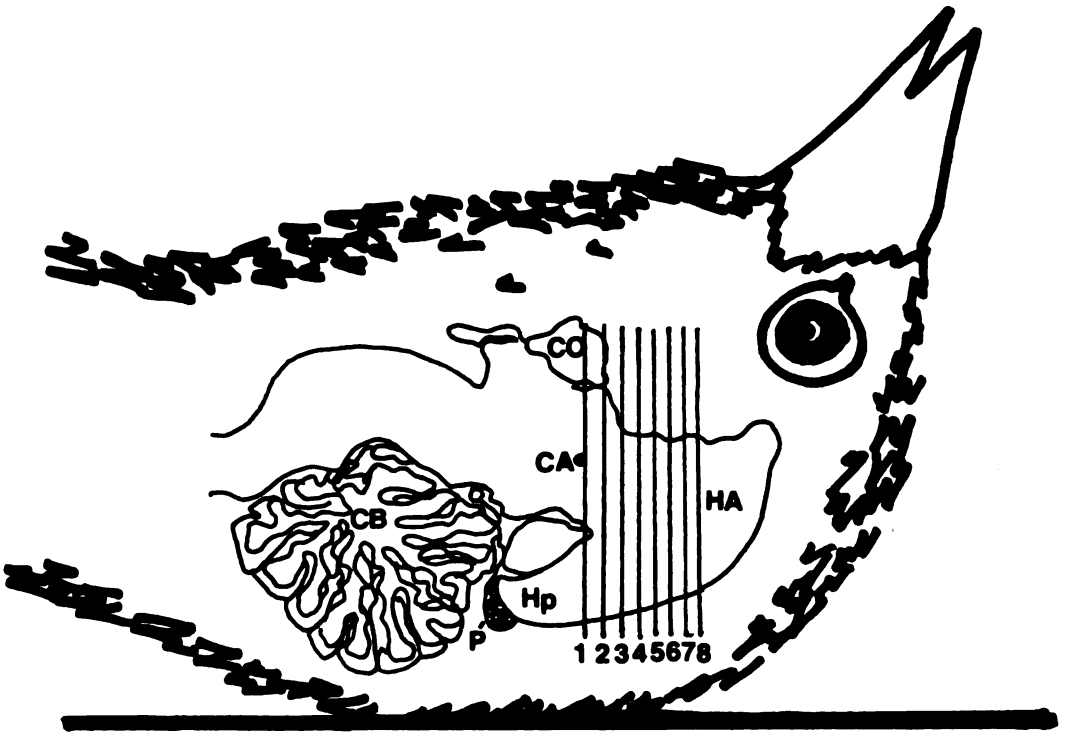


Figure 8. Illustration of CT gantry and patient scanning table

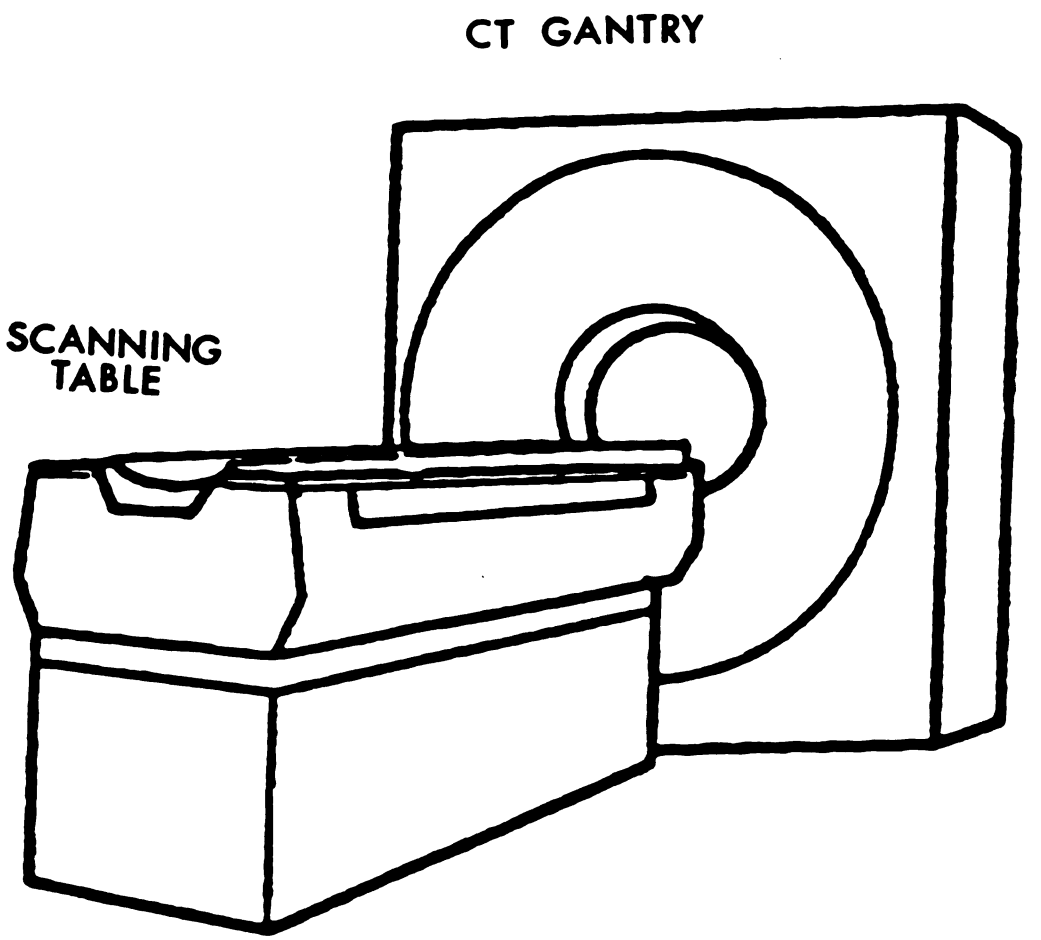


Figure 9. Printed CT image showing the cursor on the midline of the cerebral hemisphere of a hatchling double-crested cormorant chick exposed *in ovo* to PCB congener 52

The white line is the scanning table which the bird was placed on. EY = eye, CH = cerebral hemispheres, FB = frontal bone, NB = nasal bone.

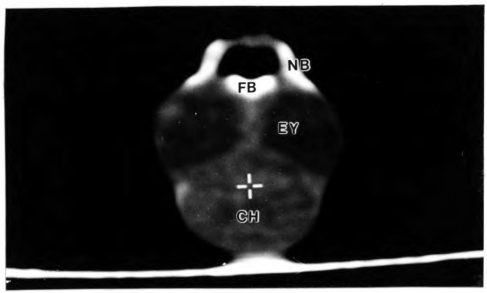


Figure 10. Printed CT image with cursor placement depicting width measurement of the cerebral hemisphere of a hatchling double-crested cormorant chick exposed *in ovo* to PCB congener 52

The line marked with the number 1 depicts the distance measured between the two cursors on the computer image. The cursor placement to obtain this measurement was based on the determined CT number range which corresponded to neural tissue.

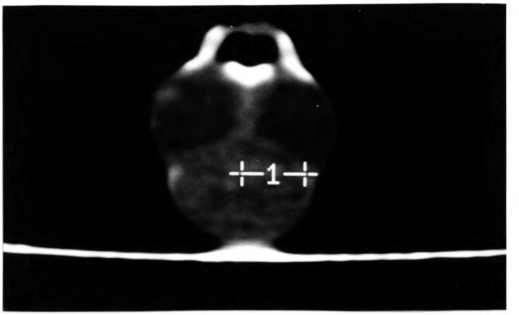


Figure 11. Printed CT image with cursor placement depicting height measurement of the cerebral hemisphere of a hatchling double-crested cormorant chick exposed *in ovo* to PCB congener 52

The line marked with the number 1 depicts the distance measured between the two cursors on the computer image. The cursor placement to obtain this measurement was based on the determined CT number range which corresponded to neural tissue.

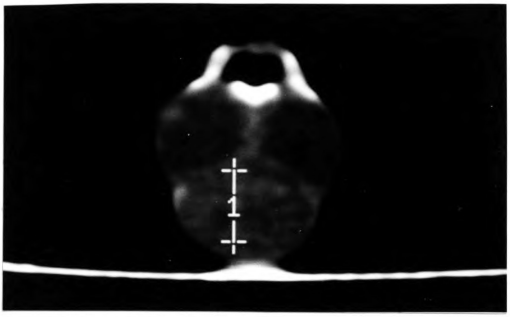


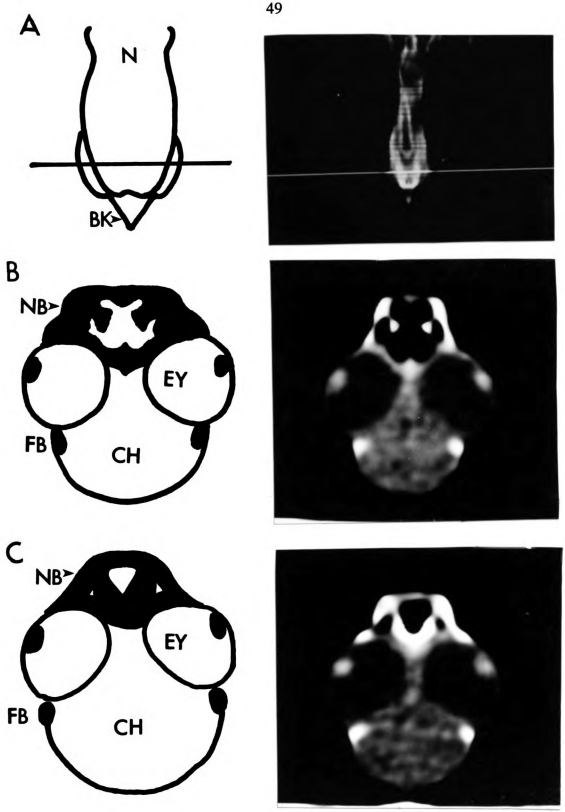
Figure 12. Printed CT images accompanied by line drawings depicting symmetrical positioning on the scanning table of the hatchling double-crested cormorant chick exposed *in ovo* to PCB congener 52

Symmetrical positioning of the bird can be assumed using the symmetry of extracranial structures as a guide. NK = neck, BK = beak, NB = nasal bone, FB = frontal bone, EY = eye, CH = cerebral hemisphere

A. Skyline view of the bird on the scanning table. The horizontal line through the bird's head is the level at which the image in Plate B is taken.

B. Coronal image of the bird head taken at the level of the horizontal line in Plate A.

C. Coronal image of the bird head taken one section level rostral (0.15 mm) to that illustrated in Plate B.



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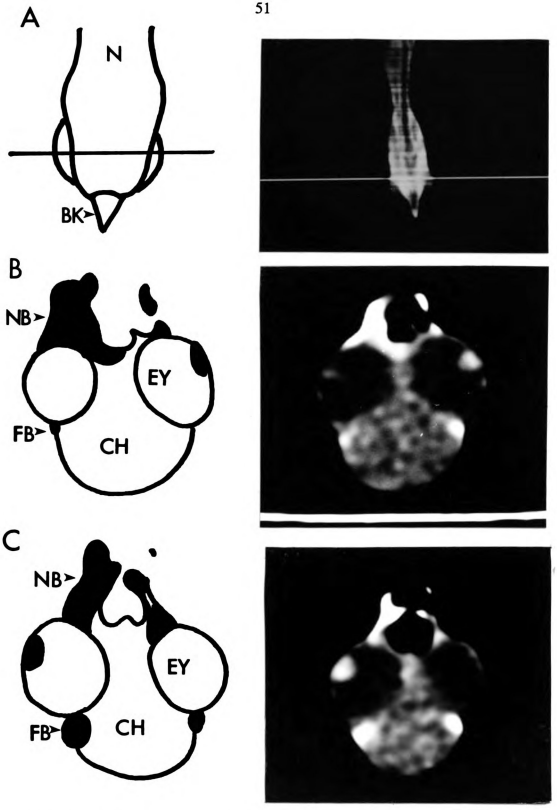

Figure 13. Printed CT images accompanied by line drawings depicting asymmetrical positioning on the scanning table of the hatchling double-crested cormorant chick exposed *in ovo* to PCB congener 52

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